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1. Introduction:

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a hereditary disorder associated with a mutation of either *PKD1* or *PKD2*. The pathophysiological mechanisms of ADPKD which ultimately result in the formation of bilateral focal cysts are not well understood. Specifically, the early disease pathways associated with the transition for a tubule to cystic phenotype are not well understood and consequently no targeted treatments for ADPKD exist. The purpose of this project is to develop and characterize a physiologically relevant platform that combines a 3D culture environment, which allows for changes in morphological phenotype, and fluid induced shear stresses that mimic the mechanical forces found in the kidney. The focus of the research was specifically targeted around developing the perfusion system and characterizing the cell types necessary for developing an *in vitro* perfusion model of ADPKD. The ultimate goal of the research is to characterize normal and disease phenotypes within the model system.

2. **Keywords:** ADPKD, perfusion bioreactor, three dimensional, tissue engineering

3. Accomplishments:

What were the major goals of the project?

Major Goals		Timeline	Percent Completion
1	Design of the perfusion system, prototyping and fabrication	1-6	100%
2	Silk scaffolding incorporation into the perfusion system	3-5	100%
3	Immortalized human renal cortical epithelial cell seeding optimization in the perfusion system with respect to flow rate, cell density and seeding duration	6-10	100%
4	Insertion of tet-pLKO-pkd1 shRNA into immortalized normal human cortical kidney cells	1-5	85%
5	Incorporation of PKD1 knockdown cells into a 3D collagen/matrigel environment	6-10	85%
6	Cyst characterization in microperfused normal and diseased (PKD1 deficient) tissues at normal and injury based flow condition	11-14	10%
7	Phenotypical and functional assessment of microperfused normal and diseased tissues at normal and injury based flow conditions	14-18	0%

What was accomplished under these goals?

- 1) *Design of the perfusion system, prototyping and fabrication:* In order to function as a model of ADPKD, the perfusion system needed to have both a 3D bulk component and physiologically relevant shear stresses across the epithelial cell surface. A primary component of the proposed perfusion system design was the inclusion of a porous silk protein scaffold capable of having extracellular matrix, specifically a 50:50 mix of 1 mg/ml type 1 collagen and matrigel, infused within the bulk. Initial bioreactor designs were focused on scaffold fabrication within rectangular wells cut out of PDMS (polydimethylsiloxane) with 250 micron wires fed across the well in order form channels within the scaffold bulk. Despite the ability to form channeled, salt-leached silk scaffolds within the wells, the porosity and channel integrity were found to be inconsistent, with low reproducibility. This design was particularly limiting considering a compromised channel structure would significantly affect the ability to accurately model the shear stresses at the epithelial cell surface. As such, we designed a novel system where a porous silk scaffold could be pre-seeded with a confluent layer of renal epithelial cells and, loaded into the top of the bioreactor and subjected to uniform shear stresses.

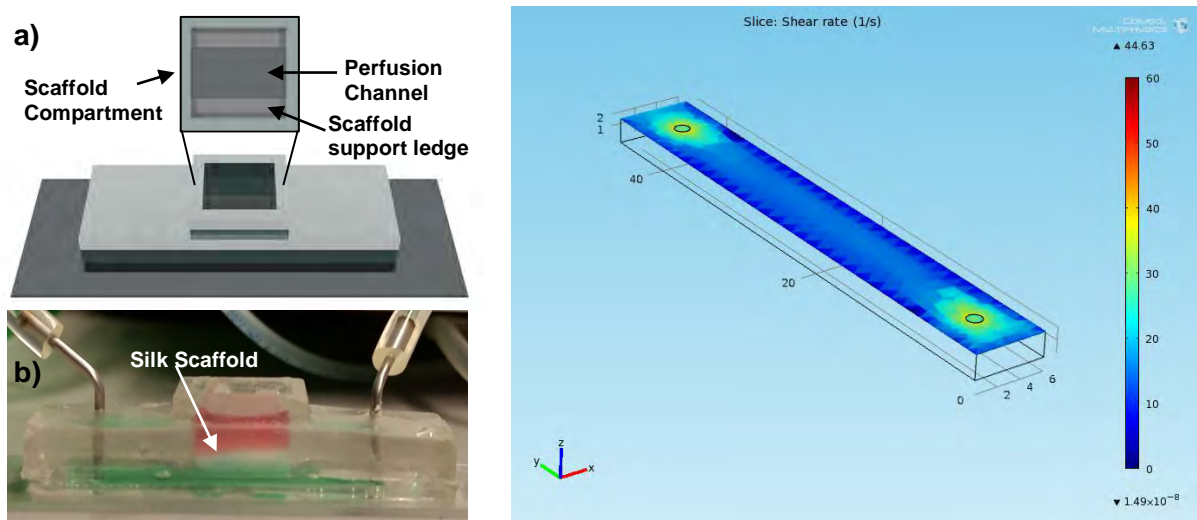


Figure 1 (a) AutoCAD model of the 3D perfusion bioreactor. The silk scaffold dimensions are several millimeters wider than the channel dimensions to allow for stable scaffold incorporation. (b) Photograph of the final assembled PDMS bioreactor. (c) COMSOL model of shear rate at the height of the scaffold within the channel.

The top loaded bioreactor was designed using AutoCAD and the system was optimized based on concurrent COMSOL modeling. The final design depicted in Figure 1 allows for the bottom of a 12 x 10 mm silk scaffold to be exposed to flow through the 44 x 6 x 2 mm channel. The bioreactor channel, port location and scaffold placement were optimized for undisrupted, laminar flow at the scaffold location. In addition to the original design parameters the final bioreactor is capable of supporting different media conditions on each side of the scaffold as depicted in Figure 1b with the green and red dyes. This capability further enhances the utility of the bioreactor to sustain complex tissue models where additional cell types and basolateral access is desirable. COMSOL modeling, as shown in Figure 1c, was used to verify uniform shear stress at the surface of the scaffold. The completed bioreactor is capable of exposing the epithelial cells to the proposed range of shear stresses that are necessary for mimicking normal and injury based flow conditions.

Repeatable device fabrication was accomplished using a custom multistep fabrication process. A negative mold of the bioreactor, designed in AutoCAD, was created using a Stratasys 3D printer. PDMS was cast in the negative molds in order to create permanent biocompatible plastic masters (SmoothCast 310).

All goals of task one were met resulting in the fabrication of a well characterized 3D perfusion bioreactor capable of reaching physiologically relevant shear stresses. Due to the dimensions of the channel high flow rates will be necessary to reach the desired shear stresses. To minimize media consumption, we have been cycling media through the system using a peristaltic pump. The resulting bioreactor is not only well suited for the proposed ADPKD tissue model but complex tissue systems requiring multiple cell types and media conditions.

- 2) *Silk scaffolding incorporation into the perfusion system:* The final bioreactor design significantly reduced the complexity of the scaffold design by eliminating the need to form a channel through the porous bulk. The channeled scaffolds were observed to have less structural integrity and the use of the wire to form the channel had a significant impact on the adjacent pores. For the current 3D perfusion system the chosen method for silk scaffold fabrication consists of freezing 5% aqueous silk at -20°C and lyophilizing. By freezing the silk solution in a 24 well plate we are able to fabricate scaffolds with a flat bottom and minimal surface defects on the side which will be exposed to flow. All scaffolds are cut to a

size of 12 x 10 x 2 mm. Scaffold pores within bulk are approximately 90 microns in diameter (Figure 2). The scaffolds are sterilized via autoclave and dried using a vacuum before infusing with Matrigel/collagen. Based on the final bioreactor design the silk scaffolds can be easily placed into the perfusion system inside the reservoir compartment and on top of the scaffold supports.

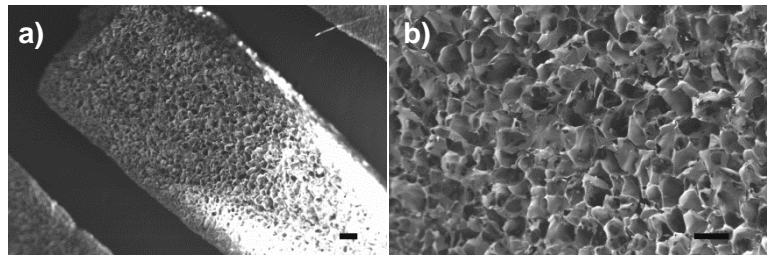


Figure 2 Scanning electron microscopy images of the cross section of a lyophilized silk scaffold. Scale bar (a) 200 microns (b) 100 microns.

- 3) *Immortalized human renal cortical epithelial cell seeding optimization in the perfusion system with respect to flow rate, cell density and seeding duration:* Similar to the scaffold optimization, the top loading bioreactor design significantly increased the reproducibility and ease of cell seeding. As opposed to most tradition perfusion systems the cells can be pre-seeded on the scaffold and subsequently placed under flow conditions. This approach significantly increases the usability of the system by eliminating the need to determine flow based seeding parameters. To establish a confluent cortical epithelial cell layer on the scaffold surface, 5×10^5 cells are added on top of each ECM infused scaffold within 12 well plates. An even layer of renal cortical epithelial cells has repeatably been established on the surface of the silk scaffold and has been shown to be viable for at least 8 weeks in static culture (Figure 3). Cell seeding can be characterized on the scaffold surface using an inverted microscope without the need for paraffin or cryo embedding.

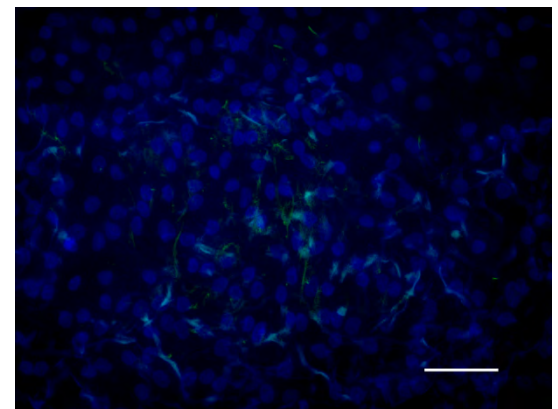


Figure 3 Renal cortical epithelial cells (RPTEC/TERT1) on the surface of the porous silk scaffold at week 8. (Blue - Dapi, Green - Acetylated alpha tubulin, Scale - 50 microns)

- 4) *Insertion of tet-pLKO-pkd1 shRNA into immortalized normal human cortical kidney cells:* To establish an inducible knockdown of *PKD1*, *PKD1* shRNA was inserted into a *pLKO-tet-on* plasmid. Plasmids were transfected into immortalized renal cortical epithelial cells (NKi-2). A range of doxycycline concentrations were dosed on the cells for 48 hours to test for induction of the knockdown. qPCR analysis revealed a reduction in *PKD1* in all concentrations between 50 and 200 ng/ml (Figure 4). While a reduction in *PKD1* is observed we were unable to achieve a complete knockdown of *PKD1* expression. In order to provide a complete perspective of diseased cell behavior in response to flow we are also currently to achieve a complete knockdown *PKD1* using CRISPR/Cas9.

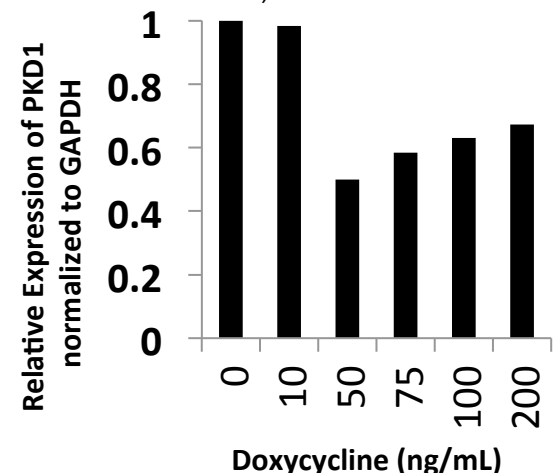


Figure 4 qPCR for *PKD1* expression 48 hours after doxycycline treatment.

- 5) *Incorporation of *PKD1* knockdown cells into a 3D collagen/matrigel environment:* Based on previously established protocols in our lab, we established the static 3D cultures of renal epithelial cells for 2

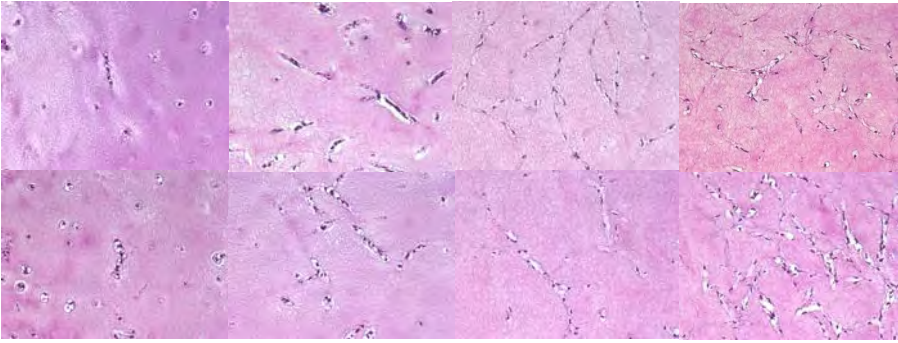


Figure 5 Renal cortical epithelial cells after 5 weeks of culture (3 weeks of treatment) at 1.0 mg/ml (a & e), 1.2 mg/ml (b & f), 1.5 mg/ml (c & g) and 1.8 mg/ml (d & h). e-h) Samples were treated with 100 ng/ml of doxycycline for 3 weeks (Scale bar – 100 microns)

weeks before induction of the knockdown. In initial studies, after 3 weeks of doxycycline treatment at concentrations of 50 and 100 ng/ml we observed a dilation of the tubular structures at the edges of the gel in the 100 ng/ml after staining with H&E (data not shown). Treatment of cultures with the cAMP agonist Forskolin did not produce notable changes in the

histology of the knockdown tissues. However, tubule dilations were observed in some of the normal cultures treated with Forskolin. Due to the localization of the phenotypic outcome to a specific region of the tissue culture it was hypothesized this difference was the result of either a higher collagen concentration or a higher stiffness at the edge of the gel. Culture optimization experiments assessing an array of collagen concentrations (1 mg/ml, 1.2 mg/ml, 1.5 mg/ml and 1.8 mg/ml) mixed 50:50 with matrigel/collagen resulted in a greater amount of tubule dilation in cultures with higher collagen concentrations (Figure 5). While complete cyst formation has not been observed within these cultures, the observed phenotypic differences upon knockdown of *PKD1* are encouraging for the proposed normal and diseased perfusion studies.

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

During the next reporting period the plan is to work on tasks 6 and 7 as outlined in the original statement of work in order to accomplish the goals and objectives of the proposal. Scaffolds will be exposed normal shear stresses ($.02-1 \text{ dyn/cm}^2$) and high flow/no flow conditions to mimic injury. The response of the tissues will be initially assessed using LIVE/DEAD and phalloidin staining to look at the extent of cell death and the integrity of the cell layer. Histological sectioning will be used to assess the extent of cell infiltration into bulk and if necessary optical sectioning using confocal imaging can also be used. While cyst formation is the desired phenotypic outcome, a lack of cyst formation in the model is not required in order to observe phenotypic differences between normal and disease conditions that can be used to elucidate the early disease mechanisms of ADPKD. Renal epithelial cells with and without the knockdown will be assessed across all conditions in order to determine differences in phenotypic and functional responses does not occur in response to knockdown the goal of assessing the functional response

4. Impact:

What was the impact on the development of the principle disciplines of the project?

The 3D perfusion platform presented here consists of a simple, modular design that is readily adaptable to a range of tissue models. This approach enables cells to be exposed to fluid forces while growing on a surface that mimics the structural environment in the body. Unlike standard two dimensional cell culture approaches the use of a 3D environment allows for the incorporation of multiple cell types. The inclusion of both the 3D protein environment and fluid flow will enable the development of more physiologically relevant tissue models for studying disease and drug development. Moreover, 3D tissue culture using this perfusion platform can be achieved with minimal technical difficulty due to ability to reliably grow cells on a structurally stable scaffold before being placed within the perfusion system. The developed 3D perfusion platform can be applied to numerous different tissue models requiring the inclusion of fluid based stimulation, such as the kidney and vasculature, and as a result the platform has the capability of having a significant translational within the field of tissue engineering. This system is currently being utilized to study the effect of fluid forces on cystic disease development within the kidney. This approach can offer new insights into possible early therapeutic treatments to limit the progression of the disease.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. Changes/Problems:

Changes in approach and reasons for change

As described above, the final bioreactor design was different from the originally proposed design due to issues with structural integrity that compromised the laminar flow through the scaffold. Despite the slight shift in design the final bioreactor design was able to achieve uniform shear stresses on the surface of a silk scaffold capable of capturing for morphological in the epithelial cell layer.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report

6. Products:

Nothing to Report

7. Participant & Other Collaborating Organizations

Nothing to Report